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## From concept to *in vivo* testing: Microcontainers for oral drug delivery

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Keywords: enteric coating; supercritical impregnation; oral drug delivery; microtechnology

## Abstract

This work explores the potential of polymeric micrometer sized devices (microcontainers) as oral drug delivery systems (DDS). Arrays of detachable microcontainers (D-MCs) were fabricated on a sacrificial layer to improve the handling and facilitate the collection of individual D-MCs. A model drug, ketoprofen, was loaded into the microcontainers using supercritical CO<sub>2</sub> impregnation, followed by deposition of an enteric coating to protect the drug from the harsh gastric environment and to provide a fast release in the intestine. *In vitro*, *in vivo* and *ex vivo* studies were performed to assess the viability of the D-MCs as oral DDS. D-MCs improved the relative oral bioavailability by 180 % within 4 h, and increased the absorption rate by 2.4 times compared to the control. This work represents a significant step forward in the translation of these devices from laboratory to clinic.

## Introduction

Oral administration of drugs is preferred by patients<sup>1</sup> due to its inherently reduced invasiveness compared to injections and minimal need for trained personnel<sup>2,3</sup>. Moreover, the effective healthcare costs can be diminished avoiding the usage of drugs administered by injections<sup>4,5</sup>.

Following oral administration, drug absorption will occur in the gastro-intestinal (GI) tract; primarily in the small intestine due to a high surface area provided by the presence of villi and microvilli<sup>6,7</sup>. When delivering drugs through the GI tract, care needs to be taken due to the presence of enzymes and a low gastric pH (1 - 3.5 in fasted state and 3 - 6 in fed state)<sup>8</sup>. Both of these can have a negative impact on the administered drug, thereby limiting the effect of the oral formulation.

Traditional oral dosage forms, such as tablets or capsules, can be designed to target the intestine. Enteric coatings can be used to protect the dosage form during transit of the stomach and facilitate the delivery of the drug to the intestinal epithelium for systemic absorption<sup>9,10</sup>. Tablets, capsules as well as micro- and nanoparticulate systems (i.e. vesicles, polymeric particles and dendrimers)<sup>11,12,13</sup>, manifest an omni-directional release of the drug in the intestinal lumen. Omni-directional release entails an inevitable loss of the drug in the lumen and therefore a reduction of the drug absorbed into the systemic circulation. Recent and promising approaches focus on reservoir-based microdevices serving as drug carriers, potentially bringing the drug to the desired place of absorption by unidirectional release from the device. An example of such microdevices is microcontainers. Here a polymeric cylinder is situated on a flat surface, defining a container structure with a cavity in the micrometer size<sup>14,15</sup>. Microcontainers provide unidirectional drug release due to their design and a protection of the drug formulation from the acidic environment of the stomach. Previously, they have been suggested as a promising oral drug delivery system, for instance maintaining indomethacin in its amorphous state<sup>16,17</sup> and being suitable for the confinement of spray dried lipid nanoparticles<sup>18</sup>. Furthermore, microcontainers have shown to improve the oral bioavailability of an amorphous salt of furosemide (a class IV drug in the Biopharmaceutics Classification System (BCS)), compared to the same drug loaded into a capsule. It was speculated by the authors that this could be due to the protection of the drug during the passage through the stomach and because of an engulfment of the microcontainers in the intestinal mucus, resulting in a prolonged absorption period<sup>19</sup>. Chirra et al. have illustrated the beneficial effect of using microdevices to improve the relative oral bioavailability of the BCS class III drug, acyclovir compared to a solution of the same drug<sup>20</sup>. Moreover, Fox et al. have shown that nanostraw membranes (porous nanostructured delivery substrates) increase adhesion to the mucus and facilitate the drug loading *via* diffusion<sup>21</sup>. So far, the presented works have only covered part of a device development and/or characterization and in most cases not reporting on *in vivo* studies, and not characterizing the devices and drugs after individual processing steps. For example, we have previously reported on drug loading of polymer filled microcontainers using supercritical carbon dioxide (scCO<sub>2</sub>)<sup>22</sup>. However, these containers were not detachable and thus, never used in animal studies. Likewise, we have reported on microcontainers loaded with powder of furosemide<sup>19</sup> where the filled containers were mechanically removed from the carrier substrate introducing a risk of damaging the containers.

Here we present, for the first time, the complete process of developing and characterizing a microcontainer-based oral delivery system. The aim of this work was to translate detachable microcontainers (D-MCs) filled with drug and sealed with a lid, from the concept to the final oral DDS suitable for *in vivo* and *ex vivo* studies. For this purpose, D-MCs have been fabricated on a sacrificial layer, improving handling and facilitating detachment and collection of the individual filled and coated D-MCs. D-MCs were loaded with the BCS class II model drug ketoprofen utilizing scCO<sub>2</sub> followed by an enteric coating to prevent release of the formulation during handling, detachment and transit through the stomach. The loaded and coated D-MCs were investigated *in vitro* to assess the functionality of the enteric lid, and *in vivo* and *ex vivo*, to evaluate the potential of microcontainers as an oral DDS.

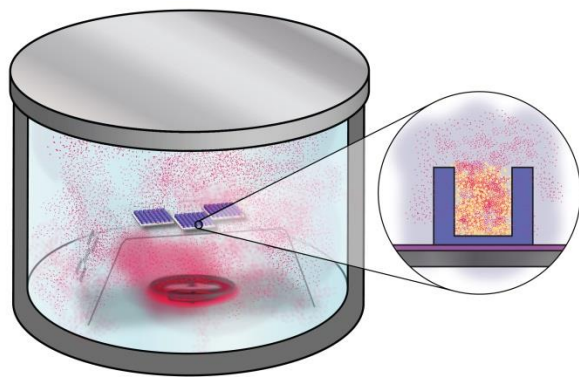
## Materials and methods

### Fabrication of detachable microcontainers (D-MCs)

Silicon (Si) wafers (4-in. b100N n-type) were supplied by Okmetic (Vantaa, Finland). SU-8 2075 and SU-8 developer were purchased from Microresist Technology GmbH (Berlin, Germany). Polyacrylic acid 35 wt % aqueous solution (PAA, Mw 100,000) was purchased from Sigma Aldrich (St. Louis, USA) and neutralized with NaOH. A 15  $\mu\text{m}$  thick film of PAA was spin coated on a Si wafer and dried at 80°C for 10 min. The PAA film served as a water soluble release layer after completed detachable microcontainers (D-MCs) fabrication<sup>23</sup>. D-MCs were fabricated with epoxy-based photoresist SU-8 using a procedure similar to the one described earlier<sup>15,16</sup>. After fabrication, the wafers were cut into square chips containing 625 D-MCs using a laser (microSTRUCT vario, 3D Microac AG, Chemnitz, Germany). The dimensions of the D-MCs were measured using an Alpha-Step IQ Stylus Profilometer (KLA-Tencor Corporation, Milpitas, USA) and optical microscopy.

### Loading of drug formulation into the microcontainers

D-MCs sitting on a Si chip were manually loaded with polyvinylpyrrolidone (PVP) (Mw = 10,000, Sigma Aldrich, St. Louis, USA) blowing away the excess in between the D-MCs using an air gun in a similar setup as described previously<sup>18,19</sup>. The chips were weighted before and after and placed within a supercritical CO<sub>2</sub> chamber (3 chips at a time, see Figure 1), together with 14.2  $\pm$  0.1 mg (n = 15, SD) of ketoprofen powder ( $\geq$  98 % racemate, Sigma Aldrich, St. Louis, USA). The impregnation of the polymer was conducted by bringing CO<sub>2</sub> over its supercritical state at 100 bar and 40 °C, keeping it under stirring for 1 h. **During this process ketoprofen solubilizes in supercritical CO<sub>2</sub> and diffuses into the polymer matrix.** The pressurization and depressurization rate were 3.9 bar/min and 2.5 bar/min, respectively.



**Figure 1: Schematic representation of the supercritical CO<sub>2</sub> impregnation process.** Within the chamber the loading of three D-MCs chips due to the solubilization of ketoprofen in the supercritical CO<sub>2</sub> is depicted. On the right, a zoom in of one D-MC during the loading process is represented.

### Enteric coating deposition

A pH sensitive polymer, Eudragit® L100 (Evonik, Darmstad, Germany) was employed for the enteric coating on the cavity of the D-MCs. A solution of 2 % w/v Eudragit® L100 and 5 % w/w in relation to the polymer of dibutyl sebacate (Sigma Aldrich, St. Louis, USA) was dissolved in isopropanol (Sigma Aldrich, St. Louis, USA).

The solution was sprayed over a chip of drug-loaded microcontainers using an ultrasonic spray coater equipped with an accumist nozzle operating at 120 kHz (Sono-Tek, USA). During the procedure, the flow rate was kept at

0.1 mL/min, together with a 1.5 W generator power. The shaping air was set to 0.02 bar, and the speed of the nozzle was maintained at 5 mm/sec, keeping a distance between the tip and the sample of 6.5 cm. The nozzle of the spray coater was positioned above the chip containing loaded D-MCs, following a path in the x-y axis to cover an area defined by the corners of the chip, previously identified using an integrated camera. Each chip was coated with two alternating wavy line spray paths having an offset of 2 mm, resulting in a total of 100 passages. The chips were kept at 40°C during the spray coating process.

### Morphology characterization

X-ray micro computed tomography (X-ray  $\mu$ CT, Zeiss Xradia 410 versa, Pleasanton, USA) was applied to assess the filling level of the ketoprofen:PVP formulation into the D-MCs and the coating morphology on the cavity of the D-MCs. The 3D tomographic reconstruction was done with the software, provided with the system, based on a FDK algorithm<sup>24</sup>. The chip with D-MCs was investigated using a high voltage of 60 kV and having an effective pixel size of 19.33  $\mu$ m, taking 1601 projection images. For examining smaller parts of the chip with a higher resolution 60 kV as high voltage and an effective pixel size of 3.02  $\mu$ m with 3201 projection images was utilized. Three areas from each sample were analyzed to obtain a more representative image of the whole chip. Capsules filled with D-MCs were scanned to assess the effect of the collection of the D-MCs after their detachment, to assess if they were separated one to each other and if the coating was still intact. For this purpose, scans were recorded with a voltage of 40 kV with a pixel size of either 10.23  $\mu$ m or 3.36  $\mu$ m, taking 1601 projection images.

The quality of both the loading and the coating of the D-MCs was investigated using a Zeiss Supra 40VP Field Emission Scanning Electron Microscope (SEM, Carl Zeiss Microscopy GmbH, Jena, Germany). The samples were placed over metallic holders and tilted to 30° prior the analyses, both low and high vacuum modes were used with a variable energy between 4 and 8 keV.

The coating thickness of Eudragit® L100 was measured by contact profilometry (Alpha-Step IQ Stylus Profilometer, KLA-Tencor Corporation, Milpitas, USA). Eudragit® L100 films were sprayed on a SU-8 covered flat silicon chip as described in the above section 'Enteric coating deposition'. The profiles were measured using a 15.6 mg tip force with a scan speed of 20  $\mu$ m/sec and a sampling rate of 50 Hz.

### Solid state characterization of ketoprofen

X-Ray powder diffraction (XRPD) was used to determine the solid state form of ketoprofen in the D-MCs and of the controls. An X'Pert PRO X-ray diffractometer (PANalytical, Almelo, The Netherlands, MPD PW3040/60 XRD; Cu KR anode,  $\lambda = 1.541 \text{ \AA}$ , 45 kV, 40 mA) was utilized. A starting angle of 5° 2 $\theta$  and an end angle of 25° 2 $\theta$  were employed for the scans with a scan speed of 0.67335° 2 $\theta$  /min and a step size of 0.0262606° 2 $\theta$ . Data were collected using X'Pert Data Collector software (PANalytical B.V.). The diffractogram of loaded and coated D-MCs was compared to that of crystalline ketoprofen, coated D-MCs loaded with crystalline ketoprofen and D-MCs loaded with 1:4 crystalline ketoprofen:PVP. The diffractograms of D-MCs on the PAA layer, PVP and Eudragit® L100 were also investigated for comparison (data not shown). Moreover, XRPD was used to verify the amorphous form of ketoprofen in the control samples for the *in vivo* studies (described in the 'Capsules preparation' section).

In addition, the solid state form of ketoprofen impregnated into the D-MCs and of control formulations was assessed by means of Raman spectroscopy using a DXR Raman microscope (Thermo Fisher Scientific, Inc., Waltham, USA). The microscope was coupled to a single grating spectrometer with 5  $\text{cm}^{-1}$  FWHM spectral resolution and  $\pm 2 \text{ cm}^{-1}$  wavenumber accuracy. All spectra were collected using a laser with a wavelength of 780 nm, with a 50 $\times$  objective and an estimated laser spot of 3.6  $\mu$ m diameter. A 50  $\mu$ m slit was utilized when analyzing bulk powder, whereas a 25  $\mu$ m pinhole was deployed to analyze the ketoprofen inside the microcontainers the laser power was equal to 10 and 20 mW, respectively. The spectra of: i) pure ketoprofen, ii) pure PVP and iii) microcontainers filled with PVP and impregnated with ketoprofen were compared.

### Release of ketoprofen from D-MCs

The efficacy of the coating and its resistance after the detachment of the D-MCs was evaluated determining the release of the impregnated ketoprofen, both in a Fasted State Simulated Gastric Fluid (FaSSGF pH 1.65 – Biorelevant<sup>®</sup>, London, UK) and Fasted State Simulated Intestinal Fluid (FaSSIF pH 6.5 - Biorelevant<sup>®</sup>, London, UK). Impregnated chips either coated or uncoated were individually immersed in 2 mL of deionized water (pH 3.25) to allow the solubilization of the PAA layer (avoiding the coating to dissolve) and hence, the detachment of the D-MCs. Suspended D-MCs were transferred into dialysis bags (MW cut off: 14,000) and placed in 20 mL of FaSSGF in an orbital shaking water bath at 37 °C, 150 rpm (Grant Instrument Ltd, model OLS26, Cambridge, UK) for 2 h. Afterwards, the bags were removed, rinsed with FaSSIF and placed in 20 mL of fresh FaSSIF at 37 °C, 150 rpm for 6 h. 20  $\mu$ L were collected at 0, 5, 10, 20, 40, 60, 120 min during the release in FaSSGF and after 1, 2, 5, 10, 20, 40, 60, 120, 240 and 360 min during the release in FaSSIF. Samples were analyzed using the UV-Vis spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific Inc., UK) at 258 nm. The amount of ketoprofen loaded in the D-MCs chips was also investigated as described in the section ‘*In vitro* release of ketoprofen from coated D-MCs’. The release curves were performed at least in triplicates (n = 3 for the coated and n = 6 for the uncoated D-MCs).

### Capsules preparation

Three chips of D-MCs were impregnated together and coated individually as described above. The solubilization of the sacrificial layer and the subsequent detachment from the Si chip were obtained soaking the chips into 5 mL of deionized water (pH 3.25). After 5 min, the water was removed and the D-MCs were dried at 37 °C for 15 min. Gelatin capsules (Torpac<sup>®</sup> size 9, Fairfield, USA) were filled with individual D-MCs ( $258 \pm 31$  D-MCs per capsule, as visible in Figure S5 in the Supplementary Information) and weighted before and after filling. The concentration of ketoprofen in the capsules was assessed *in vitro* by placing 14 capsules in 20 mL of phosphate buffered saline (PBS, Sigma Aldrich, St. Louis, USA) and kept under stirring (150 rpm) at 37 °C for 24 h. Measurements were done through UV-Vis spectroscopy as described before at a wavelength of 258 nm.

The preparation for the control for the *in vivo* study started with a physical mixture of ketoprofen and PVP powders with the same weight ratio (1:4) as in the D-MCs. The mixture was prepared by heating it up to 120 °C on a heating plate gently mixing the two compounds during the melting of the drug. The heated mixture was immediately quenched using liquid nitrogen followed by grinding to a fine powder. The amorphous form of ketoprofen was confirmed using XRPD as previously described. Gelatin capsules were loaded with  $922.4 \pm 11.5$   $\mu$ g of the grinded powder, an amount corresponding to that of the D-MCs formulation. Subsequently, the capsules were coated with a solution of 5 % w/v Eudragit<sup>®</sup> L100 and 5 % w/w dibutyl sebacate in relation to the polymer in isopropanol. The capsules were coated by dipping half of it into the coating solution and dried for 15 min before coating the other half. This procedure was repeated three times for each capsule.

### *In vivo* and *ex vivo* studies

All animal care and experimental studies were performed according to Danish and European laws, guidelines and policies for animal housing, care and experiments at the University of Copenhagen. The *in vivo* experiment was carried out at the Department of Experimental Medicine, University of Copenhagen and approved by the local institutional Animal Welfare Committee under the license number 2015–15–0201–00454. The *ex vivo* study was performed at the Department of Pharmacy, University of Copenhagen under the license number 2016–15–0201–00892. Both studies were carried out in compliance with the Danish laws regulating experiments on animals and EC Directive 2010/63/EU.

Male Sprague-Dawley rats were housed in pairs in cages to acclimatize for a period of one week with a light/dark period of 12/12 h and a temperature of 22 °C with a relative humidity of  $55 \pm 10$  %. During this period, the rats had free access to standard pellets and water.

For the *in vivo* study the rats with a weight ranging from 373 to 436 g were randomly divided into two groups. One group was dosed with capsules loaded with D-MCs (n = 11), the second group was dosed with capsules containing the control formulation (n = 6). Both types of capsules were given using a polyurethane feeding tube (Instech Laboratories Inc., Plymouth Meeting, USA), one capsule was dosed per rat. The rats were fastened for 1 h before and after the dosing, and for the rest of period they had free access to water and standard pellets. Blood (200 µL) was sampled through the lateral tail vein at 15, 30, 45, 60, 75, 90, 120 min, 4, 6, 8 and 24 h post dosing and collected in ethylenediaminetetraacetic acid tripotassium salt dihydrate (EDTA, Sigma-Aldrich, St. Louis, USA) coated tubes. Plasma was obtained by immediately spinning the blood samples at 1,500 g for 10 min. Plasma was stored at -20 °C until further analyses.

For the *ex vivo* study, two male Sprague–Dawley rats weighting 316 and 319 g were used and were fasted 1 h prior to dosing.

Capsules filled with D-MCs (see the section ‘Capsules preparation’) were administered to the rats by oral gavage as described previously. After 90 min post-dosing, the rats were sacrificed, and opened at the *linea alba* for retrieving the stomach and small intestine. These were immediately cut open and examined for localizing the D-MCs using a stereo microscope (SteReo Discovery V8, Carl Zeiss MicroImaging GmbH, Jena, Germany).

### High Pressure Liquid Chromatography (HPLC) analysis of plasma samples

HPLC analyses were performed using a Dionex Ultimate 3000 Pump equipped with a Dionex ASI-100 Automated Sample Injector and with a UV-VIS lamp.

Ketoprofen was extracted from the plasma samples using a method described elsewhere<sup>25</sup> with minor modifications. Briefly, methanol was added in a 3:1 v/v ratio to the plasma and, after vortexing the mixtures, the samples were centrifuged at 15,000 g for 6 min and the supernatants were transferred into HPLC vials.

The HPLC was run in isocratic mode using a method already described in literature with slight modifications<sup>25</sup>. The mobile phases constituted of (A): deionized water with 1 % v/v trifluoroacetate (Sigma Aldrich, St. Louis, USA) and (B): 100 % acetonitrile (Sigma Aldrich, St. Louis, USA). The ratio of the mobile phase A:B was equal to 45:55 v/v. Samples were run over a Kinetex 5.0 µm XB-C18 100 Å, 100 x 4.6 mm column (Phenomenex ApS, Nordic Region, Værløse, Denmark) at 22 °C. The injected volume was 40 µL with a flow rate of 1 mL/min and a total run time per sample of 10 min. The absorbance was measured at 258 nm.

### Statistics

For the *in vivo* studies, all results were normalized for the averages of rat mass and of the ketoprofen dosed. To calculate the standard error for the area under the curve (AUC, Table 1), the standard error of the mean of correlated variables is used

$$SE_{AUC} = \sqrt{\frac{\sum_{i=1}^M \text{var}[A_i] + \sum_{i=1}^M \sum_{j \neq i}^M \text{cov}(A_i, A_j)}{N}}$$

Where  $A_i$  is the AUC for region  $i$ .

To calculate the average amount of PVP inside a capsule, and the associated standard error, the following formula is derived. To derive the formula, it is assumed that the amount of PVP in each D-MCs in the filling process are independently distributed.



$$SE_{\overline{PVP}} = \sqrt{\frac{1}{N_c} \left( \frac{1}{NM} \text{var}[X] \text{var}[Y] + \frac{1}{N} \text{var}[X] E[Y]^2 + \frac{1}{M} \text{var}[Y] E[X]^2 \right)}$$

Where  $N_c$  is the total amount of microcontainers per chip,  $X$  is the total amount of PVP measured  $N$  times, and  $Y$  is the number of microcontainers contained inside a capsule, measured  $M$  times.

The raw data can be found in the Supplementary Information (Figure S4).

Moreover, as the sample sizes are different the effect sizes reported in Table 1 uses the Hedges  $g$  effect size defined as  $g = (M_1 - M_2)/SD_{pooled}$  where  $SD_{pooled}$  is the weighted standard deviation of the two groups<sup>26–28</sup>.

All of the data are expressed as mean and the usage of standard deviation (SD) or standard error of the mean (SE) is defined within the text. Where appropriate, statistical analysis was carried out using Student  $t$ -tests using GraphPad Prism version 6.05.  $P$ -values below 5 % ( $p < 0.05$ ) were considered statistically significant.

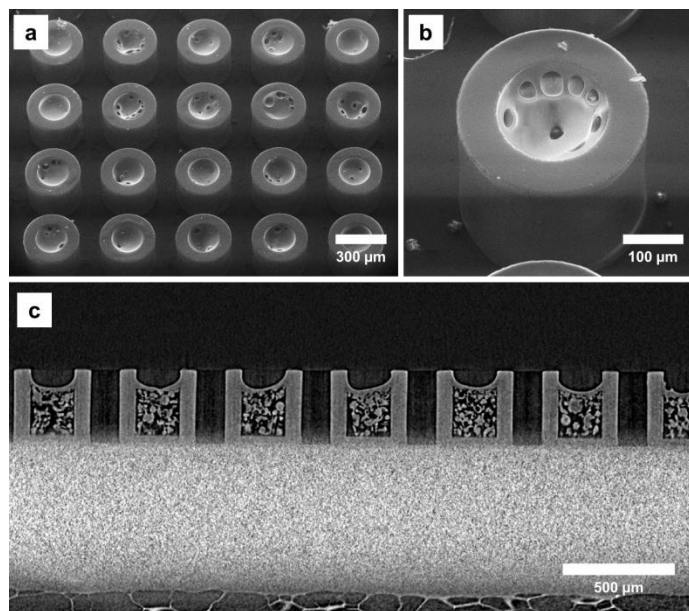
## Results

### Fabrication of microcontainers on a sacrificial layer

D-MCs were successfully fabricated in SU-8 on a water soluble layer of PAA. D-MCs had a height of  $304 \pm 12 \mu\text{m}$  ( $n = 8$ , SD) and a diameter equal to  $329 \pm 5 \mu\text{m}$  ( $n = 8$ , SD). The inner reservoir had a depth of  $272 \pm 6 \mu\text{m}$  ( $n = 8$ , SD) and a diameter of  $188 \pm 4 \mu\text{m}$  ( $n = 8$ , SD) resulting in a container volume of  $7.5 \pm 0.3 \text{ nL}$  ( $n = 8$ , SD). D-MCs were adhering well to the PAA layer not impairing the handling. D-MCs were arranged in arrays of  $25 \times 25$  devices on quadratic chips with a side length of 12.8 mm.

### Loading of D-MCs

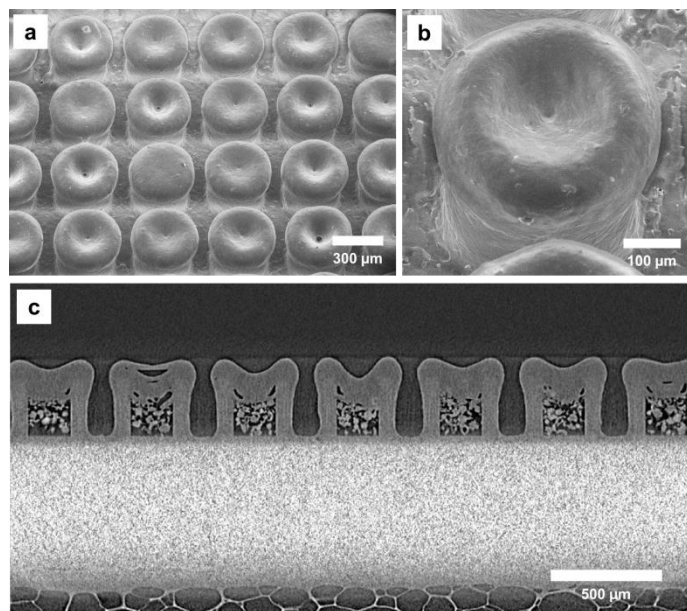
Every chip with D-MCs was manually filled with  $1.79 \pm 0.21 \text{ mg}$  ( $n = 54$  chips, SD) of PVP powder followed by loading ketoprofen into the polymer matrix using  $\text{scCO}_2$ . All chips underwent the same supercritical treatment at  $40^\circ\text{C}$  and 100 bar for 1 h. The filled D-MCs on chips were visualized using a SEM (Figure 2a, b). The cross-sectional X-ray  $\mu\text{CT}$  image of the D-MCs loaded with PVP and ketoprofen is shown in Figure 2c.



**Figure 2: Morphological characterization of drug loaded D-MCs.** (a) and (b): SEM images of D-MCs first manually loaded with PVP and then impregnated with ketoprofen in supercritical CO<sub>2</sub> at 40 °C and 100 bar for 1 h. (c): X-ray µCT cross-sectional view of the loaded D-MCs.

### Enteric coating deposition onto drug-loaded D-MCs

The spray coated gastro-resistant lid of Eudragit® L100 was, initially, characterized using contact profilometry to define the coating thickness on two chips. This resulted in thicknesses of  $123.0 \pm 1.9$  and  $118.7 \pm 3.3$  µm (SD describes the roughness of the surface of the coating). X-Ray µCT and SEM were utilized to assess the morphology of the coatings after their deposition on the cavity of the D-MCs. The coatings were homogenous (Figure 3a, b) and well distinguishable from the impregnated PVP and ketoprofen (Figure 3c).

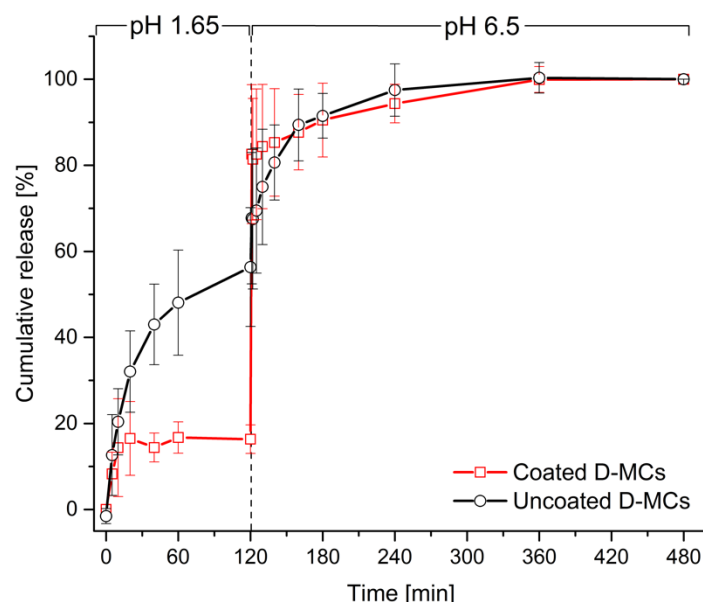


**Figure 3: Morphological characterization of loaded and coated D-MCs.** (a) and (b): SEM images of D-MCs coated with Eudragit® L100 onto the cavity of the drug-loaded D-MCs. (c): X-ray  $\mu$ CT cross-sectional view of the drug-loaded and coated D-MCs.

### ***In vitro* release of ketoprofen from coated D-MCs**

The detachment of the drug-loaded and coated D-MCs from the PAA layer was accomplished by soaking chips in deionized water at pH 3.25 for about 5 min.

The release of ketoprofen was evaluated in human FaSSGF for 120 min (simulating the residence time in the stomach) followed by investigation of the drug release in human FaSSIF for 360 min (simulating the transit time of the small intestine). After 120 min in FaSSGF,  $56 \pm 14$  % of the loaded ketoprofen from uncoated D-MCs was released compared to  $16 \pm 3$  % from the coated D-MCs (Figure 4). Upon changing to FaSSIF, a burst release with a significant immediate concentration difference was noticed for the coated microcontainers ( $p$ -value = 0.0022). After 6 h in FaSSIF, 100 % of the loaded ketoprofen was released from both the coated and uncoated D-MCs. The release profile of ketoprofen for the uncoated D-MCs did not present a burst release, but instead followed a first order kinetic. Consequently, statistical significance ( $p$ -value = 0.002) was noticeable for the release of ketoprofen after 2 h between coated and uncoated D-MCs. The total amount of ketoprofen loaded into a single chip with 625 D-MCs was  $424 \pm 10$   $\mu$ g ( $n = 14$ , SE) corresponding to a weight ratio of ketoprofen to PVP of approximately 1:4 (see the 'Formulation preparation for *in vivo* and *ex vivo* studies' section). The total amount of ketoprofen loaded into the coated and uncoated D-MCs were seen to be very similar. No significant difference was found ( $p$ -value = 0.2542).



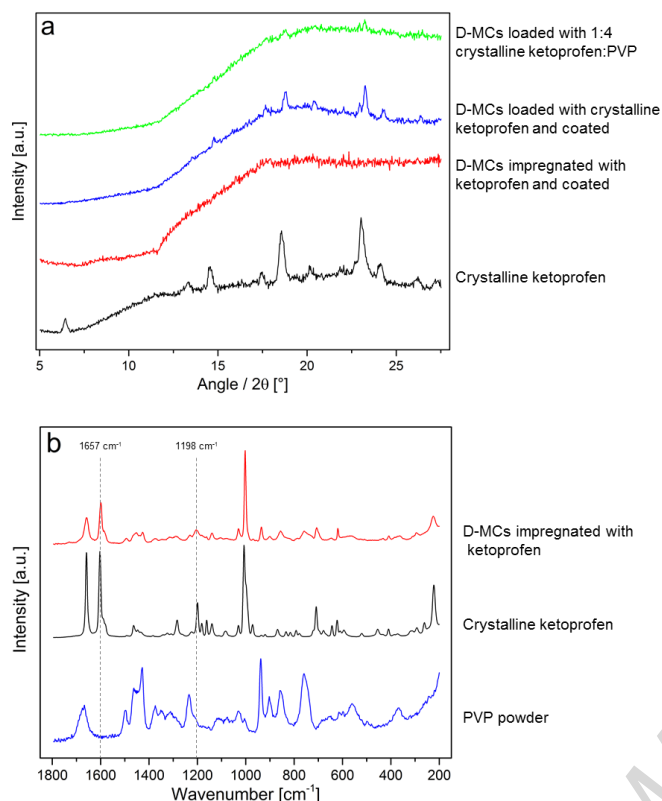
**Figure 4:** *In vitro* cumulative release of ketoprofen. Coated (red line) and uncoated (black line) D-MCs. For the first 120 min, the chips were placed in FaSSGF and subsequently in FaSSIF for 360 min. Each release curve is calculated as mean  $\pm$  standard deviation ( $n = 6$  for the uncoated D-MCs,  $n = 3$  for the coated D-MCs). For the individual profiles refer to Figure S3 in the Supplementary Information.

### Solid state characterization of ketoprofen in D-MCs

The solid state form of ketoprofen in the D-MCs both after  $\text{scCO}_2$  impregnation and after additional enteric coating deposition was evaluated by means of XRPD. By comparing the diffractograms (Figure 5a), it was found that the distinct peaks of crystalline ketoprofen were not visible in the final  $\text{scCO}_2$  impregnated and coated microcontainers. This, together with the typical scattering halo (Figure 5a, red), indicated the maintenance of ketoprofen in its amorphous form within the D-MCs. The two controls (coated D-MCs loaded with crystalline ketoprofen and D-MCs with a crystalline ketoprofen:PVP mixture in the ratio 1:4) demonstrated that it was possible to measure through the coating and to detect crystalline ketoprofen in the D-MCs in the same quantity as seen in the  $\text{scCO}_2$  impregnated and coated microcontainers (Figure 5a, blue and green).

It is worth mentioning that the melted and quenched mixture of ketoprofen:PVP 1:4 (used as control for the *in vivo* studies) was also found to be amorphous in the XRPD diffractograms (see in Supplementary Information Figure S1).

The XRPD results were corroborated by Raman spectroscopy comparing the spectra of pure crystalline ketoprofen, pure PVP and microcontainers filled with PVP and impregnated with ketoprofen (Figure 5b). As noticeable from the Raman spectra, the characteristic vibrational patterns of ketoprofen were also visible in the impregnated D-MCs. Briefly, the intensity of the peak at  $1657\text{ cm}^{-1}$ , which is attributed to the vibrational stretch of the carbonyl  $\nu(\text{C}=\text{O})$ , decreased compared to that of crystalline ketoprofen. Moreover, the broadening of the band around  $1198\text{ cm}^{-1}$  (CH ring plane bending) together with the lowering of the peak intensities between  $1500\text{ cm}^{-1}$  and  $1100\text{ cm}^{-1}$ , supported the hypothesis of ketoprofen amorphization due to the impregnation process<sup>29</sup>.



**Figure 5: Solid state characterization of ketoprofen in D-MCs.** (a) XRPD diffractograms of crystalline ketoprofen (black), D-MCs impregnated with ketoprofen and coated (red), D-MCs loaded with crystalline ketoprofen and coated (blue) and D-MCs loaded with 1:4 crystalline ketoprofen:PVP (green). (b) Raman scattering profiles of PVP powder (blue), crystalline ketoprofen (black) and D-MCs impregnated with ketoprofen (red).

### Formulation preparation for *in vivo* and *ex vivo* studies

Gelatin capsules were filled with  $258 \pm 31$  ( $n = 54$ , SE)  $\text{scCO}_2$  loaded and coated D-MCs corresponding to  $176 \pm 14$   $\mu\text{g}$  ( $n = 14$ , SE) of ketoprofen and  $741 \pm 52$   $\mu\text{g}$  ( $n = 54$ , SE) of PVP.

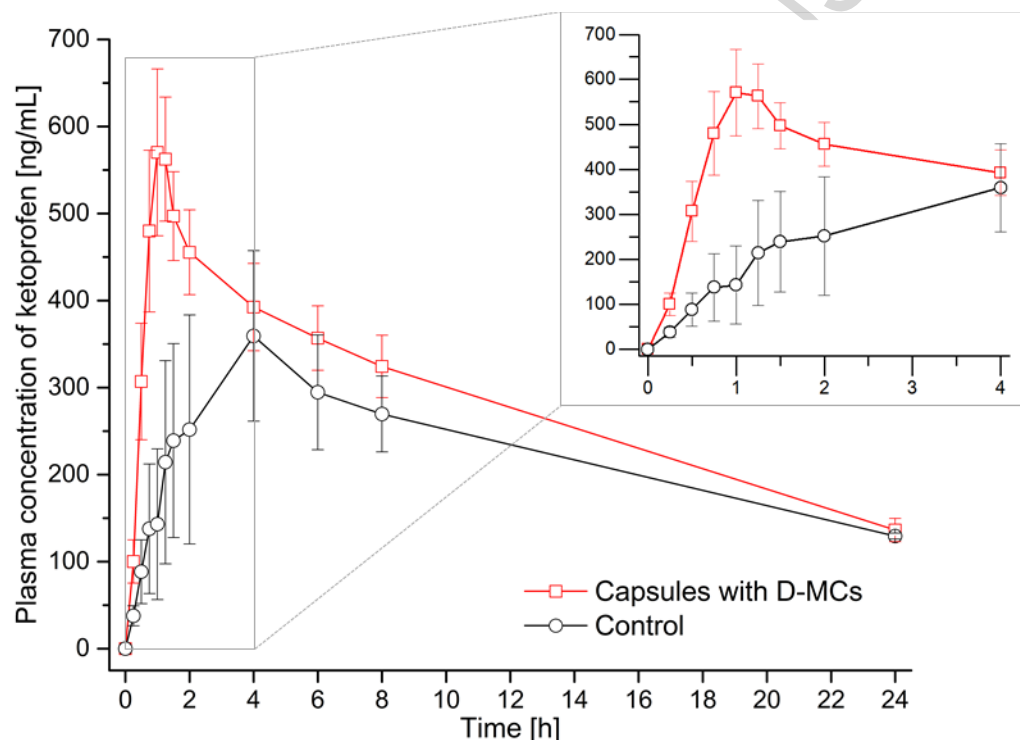
X-ray  $\mu\text{CT}$  was employed to visualize the microcontainers inside the capsule. It can be seen that the coating was preserved through all preparation steps and that the microcontainers were intact and separated from each other (Figure 6).



**Figure 6: X-ray  $\mu\text{CT}$  image of a gelatine capsule filled with loaded and coated D-MCs.**

### *In vivo studies*

Capsules filled with D-MCs or with the control formulation were dosed by oral gavage to rats. The measured plasma concentration of ketoprofen over time is presented in Figure 7 and key results are summarized in Table 1. The maximum plasma concentrations ( $C_{max}$ ) are similar for D-MCs and the control. The values were found to be  $657 \pm 78$  ng/mL and  $488 \pm 105$  ng/mL for the formulation with D-MCs and for the control, respectively (p-value = 0.2191). The AUC from 0 to 24 h ( $AUC_{0-24h}$ ) was calculated to be  $406 \pm 40$  min•ng/mL for the D-MCs formulation and  $320 \pm 49$  min•ng/mL for the control, thereby, no significant difference was observed between the two groups (p-value = 0.2041). The relative bioavailability from 0 to 24 h for ketoprofen in D-MCs compared to the control was found to be  $127 \pm 23$  %. However, statistically relevant difference (p-value = 0.0279) was found for the time corresponding to the maximum plasma concentration ( $T_{max}$ ) when comparing the two formulations ( $93 \pm 17$  min for the D-MC and  $212 \pm 60$  min for the control). The  $AUC_{0-4h}$  for the D-MCs formulation was  $99 \pm 10$  min•ng/mL and  $55 \pm 18$  min•ng/mL for the control resulting in a statistically significant difference between the two groups (p-value = 0.0387). According to this, the oral relative bioavailability from 0 to 4 h was  $180 \pm 62$  % for the D-MC formulation compared to the control. The absorbance rate of ketoprofen ( $C_0$  to  $C_{max}$ ) for the rats dosed with D-MCs was  $10 \pm 2$  ng • min<sup>-1</sup> • mL<sup>-1</sup>, which is significantly higher than for the control ( $4 \pm 1$  ng • min<sup>-1</sup> • mL<sup>-1</sup>) (p-value = 0.0430, Figure 7, top right).



**Figure 7: Plasma concentration of ketoprofen over time.** (Red line), capsules with loaded and coated D-MCs (n = 11, SE). (Black line), control capsules filled with melted ketoprofen and PVP and coated (n = 6, SE). The inset represents the same profiles zoomed in the first 4 h. For the individual profiles refer to Figure S4 in the Supplementary Information.

**Table 1: Non-compartmental model of the *in vivo* study of ketoprofen in D-MCs (n = 11, SE) and for the control formulation (n = 6, SE).**

	Capsule with loaded and coated D-MCs	Coated capsules with 1:4 ketoprofen:PVP amorphous mixture (control)	Effect size**
$C_{\max}$ [ng/mL]	$657 \pm 78$	$488 \pm 105$	0.65
$T_{\max}$ [min]	$93 \pm 17^*$	$212 \pm 60^*$	1.24
$AUC_{0-4h}$ [min•ng/mL]	$99 \pm 10^*$	$55 \pm 18^*$	1.16
$AUC_{0-24h}$ [min•ng/mL]	$406 \pm 40$	$320 \pm 49$	0.68
Relative oral bioavailability [%]			
0 - 4 h	$180 \pm 62 \%$		
0 - 24 h	$127 \pm 23 \%$		

\*  $p\text{-value} < 0.05$

\*\*  $Effect\ size = (M_1 - M_2)/SD_{pooled}$  where  $M_1$  and  $M_2$  are the averages of the two populations and  $SD_{pooled}$  is the weighted standard deviation of the two groups.

### Ex vivo study

In order to understand the mechanism of action of the D-MCs, their position in the GI tract of the rats at  $T_{\max}$  (90 min) was assessed. No D-MCs were found in the stomach of the rats at  $T_{\max}$ , whereas many were found in the mid-jejunum (see Figure S2 in the Supplementary Information). This indicates that the enteric coating protected the formulation until the intestine was reached, where the ketoprofen was released and absorbed.

## Discussion

Micro- and nanotechnologies are enabling new possibilities in the world of oral drug delivery. It is a highly complex and multidisciplinary field with focus on fabrication and on the possibilities to integrate novel functionalities into drug delivery systems. *In vivo* studies proving their actual performances<sup>21,30-32</sup> are, however, not always carried out.

In this work, an oral DDS based on microcontainers has been further developed compared to previous ones<sup>19,33</sup>. The complete process, starting from the fabrication of the new D-MCs to the loading and coating, highlighting the subsequent results from the *in vivo* and *ex vivo* investigations, is presented.

D-MCs were filled with PVP in a simple and reproducible manner and  $scCO_2$  was used to load the D-MCs with the model drug ketoprofen with a final 1:4 weight ratio of drug to polymer. A single D-MC has a cavity of  $7.5 \pm 0.3$  nL, 178 times larger compared to other similar DDS<sup>34</sup>, and each one was loaded with  $0.68 \mu\text{g}$  of ketoprofen, which is considerably more compared to the data presented by Chirra et al. ( $1.54 \text{ ng}$ )<sup>20</sup>. The amount of



ketoprofen in a single D-MC corresponds to 1.3 % w/w of the total weight of the microdevice. The technique of scCO<sub>2</sub> impregnation was preferred over inkjet printing due to the low spotting reproducibility and low loading capacity of the printing process<sup>35,36</sup>.

In the loading process, scCO<sub>2</sub> acts as a solvent for ketoprofen, but not for PVP, which only swells<sup>22</sup>. The porosity of PVP increased during the impregnation allowing ketoprofen to access the D-MCs. CO<sub>2</sub>, in its supercritical state, has a density similar to a liquid, whereas the viscosity and diffusivity are closer to the ones of a gas. These features are exploited during the impregnation process, where ketoprofen is used in relatively high concentration and diffuses easily with the CO<sub>2</sub> into the D-MCs.

During the scCO<sub>2</sub> impregnation, a solid state transition of ketoprofen from its crystalline to its amorphous form was obtained. Ketoprofen is a BCS class II drug meaning that it has a poor solubility in water. Therefore, its aqueous solubility can be increased by exploiting the amorphous form<sup>37–40</sup>. The XRPD diffractograms and the Raman spectra (Figure 5a and b, respectively) suggested that the amorphous form of ketoprofen was present after impregnation into the D-MCs, confirming previous results<sup>33</sup>.

Ketoprofen was kept in its amorphous form for at least 7 days (data not shown) due to the use of scCO<sub>2</sub> and to its affinity with PVP<sup>29,41,42</sup>. PVP is a water soluble polymer and has unique properties in prolonging the stability of amorphous forms of drugs, thereby increasing their dissolution rate and solubility<sup>41</sup>. Microcontainers can additionally stabilize the amorphous form of drugs by spatially confining the drug molecules, leading to an improved physical stability of the amorphous drugs<sup>16,17</sup>.

In order to avoid premature release of ketoprofen, D-MCs were coated with the gastro-resistant polymer Eudragit® L100. The *in vitro* dissolution studies (Figure 4) confirmed that this polymer successfully protected the drug during transit through the gastric environment and dissolved quickly upon arrival in the small intestine (where the pH is generally above 6)<sup>19,43–46</sup>. Spray coating by an ultrasonic nozzle was selected as the technology to deposit the coating onto the cavity of the D-MCs. The morphology of PVP and ketoprofen after impregnation (Figure 2) was suitable for the coating deposition as there was still space for the coating in the top of the cavity of the D-MCs. The deposition of the lid was simple and straightforward, and has the potential of being scaled up. D-MCs were detached from the fabrication platform by soaking them into acidified water. This approach maintains the integrity of the gastro-resistant lid (Figure 6) and it is a gentler and more controlled procedure than using, for example, mechanical forces. SEM images and X-ray  $\mu$ CT scans of coated D-MCs showed that after spray coating no agglomerates of polymer were present between adjacent microcontainers and that the D-MCs were not attached to each other after dissolution of the PAA sacrificial layer (Figure 3 and 6).

The *in vitro* release of ketoprofen from the D-MCs in gastric and intestinal simulated media demonstrated the efficacy of the coating. The immediate release of  $16 \pm 3$  % of ketoprofen from the coated D-MCs in FaSSGF can be explained by the presence of small pores in the coating (Figure 3) and/or the possible variation of the polymer morphology (refer to the video in the Supplementary Information for a more detailed view). For the uncoated D-MCs,  $56 \pm 14$  % of ketoprofen was released in FaSSGF, showing that nothing efficiently hindered the drug release. Coated D-MCs showed a very significant burst release upon changing to FaSSIF due to the dissolution of Eudragit® L100 followed by a fast release of ketoprofen, together with dissolution of PVP (Figure 4).

*In vivo* studies are necessary when testing new drug delivery systems as they provide indications on possible bioavailability improvements after oral administration compared to a control formulation<sup>47,48</sup>.

The control formulation used in these studies was designed to have the same ratio of ketoprofen:PVP (1:4) and a total amount of drug and polymer as for the D-MCs formulation to obtain information on the behavior of the D-MCs. The solid state form of a drug has a large influence on the dissolution rate, and can therefore, be of great importance for the bioavailability<sup>49</sup>. It was found that ketoprofen in the D-MCs after scCO<sub>2</sub> impregnation was amorphous and consequently, the ketoprofen in the control formulation was also brought to its amorphous form. This was obtained by melting the ketoprofen together with PVP followed by a fast cooling, which is a common method for preparing the amorphous form of a drug, as reported by Enfalt et al<sup>50</sup>.



Compared to the control, the D-MCs formulation did not provide a higher  $C_{\max}$ . However, a faster  $T_{\max}$  was observed for the D-MCs formulation being roughly 2.3 times faster than the control. This significant difference resulted in a large value for the effect size (Table 1), in accordance with the classification proposed by Choen<sup>28</sup>, where the intervals 0.00 - 0.20, 0.20 - 0.50 and 0.50 - 0.80 correspond to a small, medium or large effect, respectively. This indicates that D-MCs have a large effect on the time of absorption. For the first 4 h of the plasma concentration-time profile (Figure 7), the absorption of ketoprofen was significantly higher than for the control, again resulting in a large effect size value (Table 1). This difference resulted in a relative oral bioavailability of  $180 \pm 62$  % for the first 4 h. In accordance with the difference between the  $T_{\max}$  values, the absorption rate was significantly higher for the rats administered with D-MCs compared to the control rats. This supports the conclusion that the D-MCs provided a much faster absorption of ketoprofen compared to the control. It can be hypothesized that this, to some extent, is caused by a faster gastric emptying of the rats dosed with the D-MCs compared to the control. Indeed, D-MCs were most likely released from the gelatin capsule in the stomach as no coating was applied to the entire capsule, conversely to the control formulation. From the plasma concentration curve (Figure 7), it is noticeable that after 4 h the two formulations show more similar kinetics, and the  $AUC_{0-24h}$  is not significantly different. This is reflected in the relative bioavailability of ketoprofen in the D-MCs formulation compared to the control being  $127 \pm 23$  %. Choi et al.<sup>51</sup> evaluated the intestinal absorption of a suspension of ketoprofen in rats administering a 2.3 times higher dosage compared to the one used in the present study. The authors report a higher  $C_{\max}$  ( $6.12 \pm 1.02$   $\mu\text{g/mL}$ ) and a faster  $T_{\max}$  ( $0.42 \pm 0.29$  h). Indeed, these results might be attributed to the higher dosage and to the fact that ketoprofen was dosed in a suspension form, thus, partially pre-solubilized. An important difference comparing these two studies is the plasma concentration decay over time. The decrease is slower for the D-MCs, indicating a prolonged drug release and absorption time. A possible explanation for this might be provided by the results of our *ex vivo* study where at the time in which the  $T_{\max}$  was reached ( $93 \pm 17$  min), D-MCs were spread in the small intestine, and most of them were found in the mid-jejunum embedded deep into the mucus. This pronounced engulfment might indeed have resulted in a slower release and at the same time allowed prolonged absorption of ketoprofen. It has previously been shown in intestinal perfusion studies in rats that SU-8 microcontainers have mucoadhesive properties showing i.e. a high tendency to be engulfed by the mucus<sup>19</sup>.

## Conclusions

In this work, we demonstrated that D-MCs are a promising oral drug delivery system providing a 2.3 times faster  $T_{\max}$  and a 180 % increased  $AUC_{0-4h}$  when compared to the control. These features can be of high importance as it could imply that the administered dose could be reduced. The BCS class II model drug ketoprofen was successfully loaded into D-MCs exploiting the features of  $\text{scCO}_2$  impregnation maintaining the APi in its amorphous form. Enteric coating was employed to protect the drug from the stomach environment and to release ketoprofen in the intestine, as proven by the *in vitro* study. All preparation steps are designed to be compatible with each other maintaining ketoprofen in its amorphous state. *In vivo* and *ex vivo* analyses finally show the potentials of using D-MCs as an oral drug delivery system.

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# Graphical abstract

